

111 pcts

## DESCRIPTION

## THIN ANALYZING DEVICE

5 TECHNICAL FIELD

The present invention relates to an analyzing device used in the analysis of the concentration of a specific component (such as glucose or cholesterol) in a sample liquid such as blood.

10

BACKGROUND ART

Monitoring their blood glucose level on a daily basis is very important to diabetes patients in order to manage their blood glucose. Since making frequent 15 trips to a medical facility is so inconvenient, portable, easy-to-use blood glucose measurement devices small enough to fit in the palm of the hand are used so that patients can easily measure their blood glucose by themselves and can even conveniently measure their 20 blood glucose while away from home. Blood glucose is measured with one of these blood glucose measurement devices by installing a glucose sensor, which provides an enzyme reaction site, in the blood glucose measurement device, and supplying blood (specimen) to 25 this glucose sensor.

Many glucose sensors are designed to measure the glucose concentration in a simple blood glucose

measurement device by utilizing an electrochemical process, typically amperometry or coulometry. A glucose sensor of this type comprises, for example, a pair of electrodes (working electrode and counter 5 electrode), a reagent layer, and a capillary in which this reagent layer is housed.

When amperometry is employed, for example, the working electrode and counter electrode may either be lined up next to each other in the same plane or 10 disposed to face one another, but when coulometry is employed, the working electrode and counter electrode are generally disposed to face each other. The reagent layer contains a redox enzyme and an electron mediator, with GOD commonly used as the redox enzyme, and 15 potassium ferricyanide as the electron mediator. With a glucose sensor such as this, when the specimen is supplied to the reagent layer through the capillary, an oxidation reaction of glucose, for example, is catalyzed by the redox enzyme, while a reduction 20 reaction of the electron mediator is catalyzed by this enzyme.

Blood is generally supplied to the glucose sensor as follows. The user makes an incision in the skin to produce blood, and this blood is introduced into the 25 glucose sensor. With this method, it is preferable to sample as little blood as possible in order to make the blood sampling less of a burden to the user.

Accordingly, various improvements have been studied in an effort to reduce the amount of specimen (see, for example, PCT Publication No. WO2000-509507 and US Laid-Open Patent Application 2002/0092612).

5 PCT Publication No. WO2000-509507 discloses a glucose sensor in which a working electrode and a counter electrode are disposed to face each other and separated by a distance of no more than 50  $\mu\text{m}$ , so that the glucose concentration can be measured with a small  
10 amount of sample by coulometry. This glucose sensor does allow a smaller amount of blood to be used, but since coulometry is a method in which almost all of the glucose is reacted, a problem is that measurement takes far longer.

15 In contrast, US Laid-Open Patent Application 2002/0092612 discloses a glucose sensor in which the amount of sample is 1.5  $\mu\text{L}$  or less and the measurement time is reduced to 10 seconds. With this glucose sensor, a cavity in which the working electrode,  
20 counter electrode, and reagent layer are disposed is formed between a substrate and a cover, with the distance between the substrate and cover being no more than 200  $\mu\text{m}$ . The reagent layer of this glucose sensor is immobilized and rendered water-insoluble on the  
25 surface of the working electrode in a state of containing glucose oxidase and a ferricyanide, for example.

Nevertheless, with the glucose sensor disclosed in US Laid-Open Patent Application 2002/0092612, the reduction in measurement time can hardly be considered adequate, and there is still room for improvement in 5 terms of measurement precision.

#### DISCLOSURE OF THE INVENTION

It is an object of the present invention to be able to measure concentration precisely with a very 10 small amount of sample liquid while still keeping the measurement time short.

As a result of diligent study aimed at achieving this object, the inventors arrived at the present invention upon finding that the configuration of the 15 reagent layer is one of the reasons the measurement time could not be shortened with conventional glucose sensors.

Specifically, with the reagent layer of a conventional glucose sensor, because the reagent layer 20 was immobilized on the surface of the working electrode, the reaction between the glucose and the glucose oxidase only occurred at the surface of the working electrode, so the reaction between the glucose and the glucose oxidase took a long time, and this increased 25 the measurement time. One possible way to solve this problem is to configure the reagent layer so that it will readily dissolve in the sample liquid (blood). In

this case, since an electron mediator is diffused in the sample liquid (blood), it is necessary to eliminate anything that would affect the diffusion of the electron mediator, such as the effect of the proportion 5 of solid components in the sample liquid (such as blood cell components in blood), or the effect of the temperature of the sample liquid. Also, the dissolution time will be longer, and the measurement time will increase, when a compound such as a 10 ferricyanide that has relatively low solubility in blood is used.

The inventors also learned that it is preferable to improve the following points in order to further increase measurement precision. First, when a compound 15 such as a ferricyanide that has relatively low solubility in blood is used, there is the possibility that measurement precision will be adversely affected by variance in solubility. Also, since ferricyanides have poor storage stability and readily migrate to 20 reductants during storage, there is the danger that measurement precision could decrease in this respect as well. Second, glucose oxidase has a relatively low reaction velocity with glucose (its Km (Michaelis constant) is large), so using glucose oxidase is 25 undesirable for the purposes of shortening the measurement time.

The present invention was conceived in light of

the above situation, and provides a thin analysis tool comprising a reaction space for holding a sample liquid. The reaction space is provided with a reagent portion that dissolves when the sample liquid is held in the 5 reaction space. Part of the reaction space is defined by first and second surfaces facing each other, where the first and the second surfaces are spaced from each other by a facing distance that is no greater than 45  $\mu\text{m}$ .

10 The thin analysis tool of the present invention may, for example, further comprise first and second plates facing each other and disposed apart from each other to define the reaction space. The first and second surfaces extend in a direction perpendicular to 15 the thickness direction of the first and second plates.

The thin analysis tool of the present invention may, for example, further comprise first and second electrodes that are provided on one side of the first plate, face at least partially the reaction space, and 20 are utilized to apply voltage to the sample liquid. In this case, the facing distance is defined as the minimum distance from the upper surface of the first or second electrode (corresponding to the first surface, for example) to the portion of the second plate to face 25 the upper surface of said electrode (corresponding to the second surface, for example).

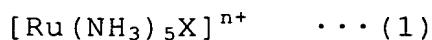
The thin analysis tool of the present invention

may, for example, further comprise a first electrode provided to the first plate, and a second electrode provided to the second plate and across from the first electrode, for applying voltage to the sample liquid 5 together with the first electrode. In this case, the facing distance is the minimum distance between the upper surface of the first electrode (corresponding to the first surface, for example) and the upper surface of the second electrode (corresponding to the first 10 surface, for example).

The reaction space may, for example, be constituted such that the sample is moved by capillary force.

The reagent portion may, for example, include an 15 electron mediator and a redox enzyme.

A ruthenium compound is preferably used as the electron mediator. The ruthenium compound can be one expressed by the following chemical formula (1).



20 In Chemical Formula 1, X is  $\text{NH}_3$ , a halogen ion,  $\text{CN}$ , pyridine, nicotinamide, or  $\text{H}_2\text{O}$ , but X is preferably  $\text{NH}_3$  or a halogen ion.  $n^+$  in Chemical Formula 1 is the valence of an oxidized Ru(III) complex determined by the type of X.

25 When the component to be analyzed is glucose, it is preferable for the redox enzyme to be GDH with glucose dehydrogenation activity. The GDH is

preferably GDH in which a cytochrome C is bonded to  $\alpha$ GDH (CyGDH). Examples of CyGDH and  $\alpha$ GDH are those disclosed in International Disclosure Pamphlet No. WO02/36779. The GDH is preferably one originating in 5 microbes belonging to the genus *Burkholderia*, but GDH originating in microbes belonging to other genera and having the same FAD and cytochrome C as CyGDH and  $\alpha$ GDH can also be used. Examples of other genera include pathogenic Gram-negative microbes among the genera 10 *Ralstonia* and *Pseudomonas*.

For example,  $\alpha$ GDH contains a GDH active protein (alpha sub unit) whose molecular weight is approximately 60 kDa as measured by SDS-polyacrylamide gel electrophoresis under reductive conditions, as a 15 sub unit having glucose dehydrogenation activity. Meanwhile, CyGDH contains two sub units: an alpha sub unit and an electron transport protein (cytochrome C) whose molecular weight is approximately 43 kDa as measured by SDS-polyacrylamide gel electrophoresis 20 under reductive conditions.  $\alpha$ GDH and CyGDH having other sub units besides an alpha sub unit and cytochrome C can also be used.

CyGDH can be obtained, for example, by purifying an enzyme externally secreted by a microbe belonging to 25 *Burkholderia cepacia*, or by purifying an internal enzyme of this microbe.  $\alpha$ GDH can be obtained, for example, by forming a transformant into which has been

transfected a gene that codes for  $\alpha$ GDH collected from a microbe belonging to *Burkholderia cepacia*, and purifying an enzyme externally secreted from this transformant, or purifying an internal enzyme of this 5 transformant.

The microbe belonging to *Burkholderia cepacia* can be, for example, *Burkholderia cepacia* KS1 strain. This KS1 strain has been deposited under microorganism accession number FERM BP-7306 with the Patent Organism 10 Depositary, National Institute of Advanced Industrial Science and Technology (Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan).

Examples of the sample liquid include blood, urine, saliva, a preparation thereof, and other such 15 biochemical samples. Examples of the component to be analyzed include glucose, cholesterol, lactic acid, and ascorbic acid.

#### BRIEF DESCRIPTION OF THE DRAWINGS

20 FIG. 1 is an overall perspective view of the biosensor according to the present invention;

FIG. 2 is a sectional view taken along the II-II line in FIG. 1;

25 FIG. 3 is an exploded perspective view of the biosensor shown in FIG. 1;

FIG. 4 illustrates the state when the biosensor shown in FIGS. 1 to 3 is mounted in a concentration

measurement device, with the biosensor shown in plan view, and the concentration measurement device as a block diagram;

5 FIG. 5A and 5B illustrate the action of the biosensor, and are sectional views of the main components of the biosensor;

FIG. 6A and 6B are sectional views illustrating another example of a biosensor;

10 FIG. 7 is a graph of the effect of the hematocrit value of blood with biosensor 1 of the present invention;

FIG. 8 is a graph of the effect of the hematocrit value of blood with biosensor 2 of the present invention;

15 FIG. 9 is a graph of the effect of the hematocrit value of blood a comparative biosensor 1;

FIG. 10 is a graph of the effect of the temperature of blood with biosensor 1 of the present invention;

20 FIG. 11 is a graph of the effect of the temperature of blood with biosensor 2 of the present invention;

FIG. 12 is a graph of the effect of the temperature of blood with comparative biosensor 1;

25 FIG. 13 is a graph of the results of evaluating the measurement range of biosensor 1 of the present invention;

FIG. 14 is a graph of the results of evaluating the reproducibility of biosensor 3 of the present invention as a time course of the response current value;

5 FIG. 15 is a graph of the results of evaluating the reproducibility of biosensor 4 of the present invention as a time course of the response current value;

10 FIG. 16 is a graph of the results of evaluating the reproducibility of a comparative biosensor 2 as a time course of the response current value; and

15 FIG. 17 is a graph of the results of evaluating the reproducibility of biosensors 3 and 4 of the present invention and comparative biosensor 2 as a time course of C.V.

#### BEST MODE FOR CARRYING OUT THE INVENTION

The best mode for carrying out the invention will now be described in specific terms through reference to 20 the drawings. In these embodiments, the description will be of a glucose sensor constituted so as to measure blood glucose levels, but the present invention is not limited to the measurement of blood glucose, and can be applied to an analysis tool that analyzes other 25 components of blood, or other sample liquids besides blood.

The glucose sensor 1 shown in FIGS. 1 to 3 is used

by being installed in a concentration measurement device 2 (see FIG. 4), and comprises a cover 5 laid over a rectangular substrate 3 with a spacer 4 in between. In this glucose sensor 1, a reaction space 6 is defined by the various elements 3 to 5. This reaction space 6 is defined as a pillar-shaped space having a rectangular cross section, moves the sample liquid introduced through an opening (introduction port) 61 by capillary force, and is able to hold the introduced sample liquid.

The spacer 4 serves to define the height of the reaction space 6, that is, the distance from the upper surface 30 of the substrate 3 to the lower surface 5a of the cover 5. In this spacer 4 is formed a slit 41 that is open at its distal end. The slit 41 serves to define the width of the reaction space 6, and the open part at the distal end of the slit 41 serves to constitute the introduction port 61 used to introduce the sample liquid into the interior of the reaction space 6.

The cover 5 has a vent opening 51. The vent opening 51 is used to vent gases inside the reaction space 6 to the outside, and communicates with the interior of the reaction space 6. Therefore, when a sample liquid has been introduced through the introduction port 61 into the reaction space 6, the capillary force produced in the reaction space 6 causes

the sample liquid to move through the inside the reaction space 6 toward the vent opening 51 formed in the cover 5.

As shown more clearly in FIG. 3, a working 5 electrode 31, a counter electrode 32, and a reagent portion 33 are formed on the upper surface 30 of the substrate 3. For the most part, the working electrode 31 and the counter electrode 32 extend in the lengthwise direction of the substrate 3. The ends 31a 10 and 32a of the working electrode 31 and counter electrode 32 extend in the lateral direction of the substrate 3, and are lined up in the lengthwise direction. The ends 31b and 32b of the working electrode 31 and counter electrode 32, meanwhile, 15 constitute terminals for contact with first and second terminals 20a and 20b (see FIG. 4) of the concentration measurement device 2 (discussed below).

The working electrode 31 and the counter electrode 32 are formed, for example, by screen printing, plating, 20 or sputtering in a thickness D (see FIG. 2) of 20  $\mu\text{m}$  or less. The thickness D of the working electrode 31 and counter electrode 32 is preferably set to between 1 and 10  $\mu\text{m}$ . The facing distance H1 (see FIG. 2) from the upper surface 31c of the working electrode 31 to the 25 lower surface 5a of the cover 5 is set to 45  $\mu\text{m}$  or less, and preferably to between 25 and 45  $\mu\text{m}$ . This is because if the facing distance H1 is too large, as will

be discussed below, the temperature or hematocrit value of the blood will tend to have an effect, but if the facing distance H1 is too small, the blood cannot be properly moved inside the reaction space 6.

5        The reagent portion 33 is formed, for example, as a solid containing a mediator (electron mediator) and a relatively small amount of redox enzyme, and as shown clearly in FIGS. 2 and 3, is provided so as to bridge the ends 31a and 32a of the working electrode 31 and 10 counter electrode 32. This reagent portion 33 readily dissolves in blood. Therefore, when blood is introduced into the reaction space 6, a liquid phase reaction system is created including a mediator, a redox enzyme, and glucose. In this liquid phase 15 reaction system, a glucose oxidation reaction and a mediator reduction reaction occur not only on the upper surface 31c of the working electrode 31, but over a wide range in the reaction space 6. Therefore, more glucose can be oxidized and in a shorter time than when 20 a mediator or redox enzyme is immobilized on the surface of a working electrode. This means that the measurement can be completed in less time.

It is preferable to use a ruthenium compound as the mediator. A ruthenium complex is an example of a 25 ruthenium compound. There are no particular restrictions on the type of ligands of the ruthenium complex as long as they function as an electron

transport, but the complex is preferably contained in an oxidized state in the reagent portion 33. For instance, an oxidized compound expressed by the following chemical formula (2) can be used.

5                    $[\text{Ru}(\text{NH}_3)_5\text{X}]^{n+} \cdots (2)$

In Chemical Formula 2, X is  $\text{NH}_3$ , a halogen ion,  $\text{CN}$ , pyridine, nicotinamide, or  $\text{H}_2\text{O}$ , but X is preferably  $\text{NH}_3$  or a halogen ion.  $n+$  in Chemical Formula 2 is the valence of an oxidized Ru(III) complex determined by 10 the type of X.

The ruthenium complex is usually present as an oxidized type (III) since the reductive type (II) is unstable. Accordingly, the mediator will not readily be reduced even if the reagent portion 33 of the 15 glucose sensor 1 is exposed to light or water in a state in which a ruthenium complex is admixed. This means that any measurement error that would otherwise be caused by exposure of the mediator can be suppressed.

Another characteristic of a ruthenium complex is that 20 it does not readily crystallize, and favorably retains its micropowder form. Accordingly, if a ruthenium complex is used, there will be no deterioration in the solubility of the reagent portion 33 during storage. Another advantage regarding the combination of a 25 ruthenium complex with  $\alpha\text{GDH}$  or  $\text{CyGDH}$  is that the measurement time can be shortened because the electron transport rate of a ruthenium complex is high.

Meanwhile, it is preferable to use the above-mentioned  $\alpha$ GDH or CyGDH as the redox enzyme. These enzymes have the advantage of a higher reaction velocity with glucose than glucose oxidase. This also 5 affords a decrease in measurement time.

As shown in FIG. 4, the concentration measurement device 2 comprises the first and second terminals 20a and 20b, a voltage application portion 21, a current value measurement portion 22, a detection portion 23, a 10 control portion 24, a computation portion 25, and a display portion 26.

The first and second terminals 20a and 20b serve to provide contact with the ends 31b and 32b of the working electrode 31 and counter electrode 32 in the 15 glucose sensor 1 when the glucose sensor 1 has been installed in the concentration measurement device 2.

The voltage application portion 21 is utilized in the application of voltage between the working electrode 31 and the counter electrode 32 of the 20 glucose sensor 1 via the first and second terminals 20a and 20b. A dry cell, chargeable cell, or other such DC power supply is used, for example, as the voltage application portion 21.

The current value measurement portion 22 is used 25 to measure, as the response current value, the amount of electrons accepted between the working electrode 31 and the mediator during the application of voltage to

the working electrode 31 and the counter electrode 32.

The detection portion 23 is used to confirm whether or not the sample liquid has been supplied to the reagent portion 33 (see FIGS. 1 to 3) on the basis 5 of the current value measured by the current value measurement portion 22, after the glucose sensor 1 has been installed in the concentration measurement device 2.

The control portion 24 controls the voltage 10 application portion 21 and selects whether voltage will be applied (closed circuit) or will not be applied (open circuit) between the working electrode 31 and the counter electrode 32.

The computation portion 25 is used to compute the 15 glucose concentration according to the response current value measured by the current value measurement portion 22. The computation portion 25 is designed to be able to compute the glucose concentration by an amperometric method, for example. Using an amperometric method 20 allows the concentration to be measured in less time than employing a coulometric method.

The detection portion 23, control portion 24, and computation portion 25 are each constituted by a CPU and a ROM, RAM, or other such memory, for example, but 25 it is also possible for the detection portion 23, control portion 24, and computation portion 25 all to be constituted such that a plurality of memories are

connected to a single CPU.

The display portion 26 is used to display the results of the computation by the computation portion 25, and to display that an error has occurred, the 5 operating procedure, and so forth, and is constituted by a liquid crystal display device, for example.

The procedure by which glucose concentration is measured using the glucose sensor 1 and the concentration measurement device 2 will now be 10 described.

As shown clearly in FIG. 4, first, the glucose sensor 1 is installed in the concentration measurement device 2. This brings the ends 31b and 32b of the working electrode 31 and counter electrode 32 into 15 contact with the first and second terminals 20a and 20b of the concentration measurement device 2. In this state, it is possible to apply voltage between the working electrode 31 and counter electrode 32 via the first and second terminals 20a and 20b. In actual 20 measurement, a constant voltage is applied between the working electrode 31 and counter electrode 32 from the point when the glucose sensor 1 is installed in the concentration measurement device 2. Since a ruthenium complex performs mediation at a low voltage, when a 25 ruthenium complex is used, the constant voltage applied between the working electrode 31 and the counter electrode 32 is set to within a range of 100 to 500 mV,

for example. In this embodiment, the application of constant voltage between the working electrode 31 and the counter electrode 32 is performed continuously until the response current value is measured for the 5 computation of the glucose concentration.

Next, blood is introduced through the introduction port 61 of the glucose sensor 1 into the reaction space 6. The blood proceeds by capillary force from the introduction port 61, toward the vent opening 51 formed 10 in the cover 5, and into the reaction space 6. The blood dissolves the reagent portion 33 in the course of its movement.

Once blood has been supplied to the reagent portion 33, the glucose is oxidized by the redox enzyme 15 into gluconolactone, and the mediator becomes reductive. Gluconolactone non-enzymatically becomes gluconic acid.

The reductive mediator moves to the end 31a side of the working electrode in a state in which a constant voltage has been applied to the working electrode 31 20 and the counter electrode 32 through the ends 31b and 32b of the working electrode 31 and counter electrode 32, then releases electrons to this end 31a and becomes an oxidative mediator. Therefore, when a constant voltage has been applied between the working electrode 25 31 and the counter electrode 32 by the voltage application portion 21, the amount of electrons imparted from the reductive mediator is measured as the

response current value by the current value measurement portion 22 via the working electrode 31 and the first terminal 20a. This response current value is a function of the amount of electrons originating in the 5 reductive mediator that has moved through the reagent portion 33 under voltage application, and is called diffusion current.

Meanwhile, the response current value measured by the current value measurement portion 22 is monitored 10 by the detection portion 23, and at the point when the response current value exceeds a certain threshold, the detection portion 23 detects that blood has been supplied to the reagent portion 33 and the reagent portion 33 has been dissolved. When the detection 15 portion 23 detects the supply of blood, the detection portion 23 then decides whether or not a specific amount of time has elapsed since this detection.

When the detection portion 23 has decided that the specified time has elapsed, the current value 20 measurement portion 22 measures the response current value, and the computation portion 25 computes the glucose concentration. The glucose concentration is computed by converting the response current value into a voltage value, and then plugging this voltage value 25 into a previously produced calibration curve indicating the relation between voltage values and glucose concentrations. The computational result from the

computation portion 25 is displayed on the display portion 26, for example.

The reductive mediator in contact with the working electrode 31 instantly becomes oxidative upon releasing 5 its electrons to the working electrode 31, and even when the reductive mediator is a specific distance away from the working electrode 31, it will still release its electrons to the working electrode 31 and become oxidative. Hereinafter, the region in which the 10 reductive mediator is able to release its electrons to the working electrode 31 will be referred to as the electron release region, and the region in which the reductive mediator is unable to release its electrons to the working electrode 31 will be referred to as the 15 electron non-release region.

As can be surmised from the working examples given below, the distance from the surface of the working electrode in the electron release region is never less than 45  $\mu\text{m}$ . Therefore, as shown in FIG. 5A, if the 20 facing distance  $H1$  from the upper surface 31c of the working electrode 31 to the lower surface 5a of the cover 5 is relatively large, such as when the facing distance  $H1$  is at least 50  $\mu\text{m}$ , the electron non-release region 71 will be above the electron release region 70 25 directly over the working electrode 31.

In contrast, when the facing distance  $H1$  from the upper surface 31c of the working electrode 31 to the

lower surface 5a of the cover 5 is set to be 45  $\mu\text{m}$  or less, as with the glucose sensor 1 of the present invention, as shown in FIG. 5B, the thickness of the portion of the electron release region 70 located 5 directly over the working electrode 31 (hereinafter referred to simply as the "thickness of the electron release region 70") coincides with the facing distance  $H_1$ , and this thickness of the electron release region 70 will be the same as or less than that shown in FIG. 10 5A.

Thus, the situation directly over the working electrode 31 is different when the facing distance  $H_1$  is large (see FIG. 5A) and when it is small (see FIG. 5B). As a result, as can be surmised from the working 15 examples of the present invention given below, how much of the reductive mediator is consumed will vary with the facing distance  $H_1$ .

Let us assume here that when no voltage is being applied, the concentration of the reductive mediator 20 present in the electron release region (hereinafter referred to as the "non-diffused mediator") is the same as the concentration of the reductive mediator present in the electron non-release region (hereinafter referred to as the "diffused mediator").

25 In the case shown in FIG. 5A, when the facing distance  $H_1$  is large, the thickness of the electron release region 70 (the portion surrounded by the dotted

line) is also large, so not all of the non-diffused mediator is oxidized when voltage is applied. Therefore, the non-diffused mediator is consumed a specific amount at a time, and this causes a difference 5 in the concentration of the reductive mediator between the electron release region 70 and the electron non-release region 71. Consequently, the diffused mediator spreads out above and to the sides of the electron release region 70. After this, the oxidation of the 10 reductive mediator present in the electron release region 70 occurs concurrently with the spreading of the diffused mediator with respect to the electron release region 70. Therefore, when the facing distance  $H_1$  is large, the process can be broadly divided into an early 15 phase of consumption of the non-diffused mediator, a middle phase of consumption of the non-diffused mediator and the diffused mediator, and a late phase of consumption of the -diffused mediator.

The diffusion rate of the diffused mediator here 20 is affected not only by the difference in the concentration of the reductive mediator between the electron release region 70 and the electron non-release region 71, but also by the temperature and movement resistance (blood hematocrit) of the diffusion medium 25 (blood). Therefore, when the facing distance  $H_1$  is large, the effect of temperature and hematocrit value of the blood gradually increases over time.

In contrast, when the facing distance  $H_1$  is small (see FIG. 5B), because the electron release region 70 is not very thick, nearly all of the non-diffused mediator is consumed in the early phase, and then the 5 diffusion and consumption of the diffused mediator occur in the electron release region. Therefore, when the facing distance  $H_1$  is small, the process can be broadly divided into an early phase of consumption of the non-diffused mediator, and a late phase of 10 consumption of the diffused mediator. Therefore, when the facing distance  $H_1$  is small, there is a stage in which the temperature and hematocrit value of the blood have less effect, and a stage in which they have more effect.

15 When the facing distance  $H_1$  is the same as the thickness of the electron release region, the diffusion of the diffused mediator in the electron release region proceeds only from the sides of the electron release region. Accordingly, we can conclude that the 20 diffusion rate of the diffused mediator and so forth have less effect on the measured current value when the facing distance  $H_1$  is small than when the facing distance  $H_1$  is larger than the thickness of the electron release region and the diffused mediator is 25 diffused from the sides and from above the electron release region. The behavior of the diffused mediator has particularly little effect on the measured current

value in the stage when the blood temperature and hematocrit value have less effect. Therefore, if the facing distance  $H1$  is made about the same as or smaller than the thickness of the electron release region, the 5 blood temperature and hematocrit value will have less effect and reproducibility will be good in a short time span from the start of voltage application (a shorter span of measurement time).

10 The glucose sensor according to the present invention is not limited to the embodiment given above, and various design modifications are possible. For instance, the working electrode 31 and the counter electrode 32 may face at least partially the reaction 15 space 6. For example, the configuration shown in FIGS. 6A and 6B can be employed.

The glucose sensor 1' shown in FIG. 6A comprises recesses 35' and 36' formed in a substrate 3', and a working electrode 31' and a counter electrode 32' 20 embedded in these recesses 35' and 36'.

The upper surfaces 31c' and 32c' of the working electrode 31' and counter electrode 32' may or may not be in the same plane as the upper surface 30' of the substrate 3' (shown in the same plane in the drawings).

25 With this glucose sensor 1', the facing distance  $H1'$  is defined as the distance from the upper surface 31c' of the working electrode 31' to the lower surface

5a' of the cover 5', and when the upper surfaces 31c' and 32c' of the working electrode 31' and counter electrode 32' are in the same plane as the upper surface 30' of the substrate 3', the facing distance 5 H1' coincides with the distance H2' between the substrate 3' and the cover 5'.

Meanwhile, the glucose sensor 1" shown in FIG. 6B comprises a working electrode 31" formed on a substrate 3", and a counter electrode 32" formed on a cover 5".  
10 Naturally, the counter electrode may instead be formed on the substrate, and the working electrode on the cover.

With this glucose sensor 1", the facing distance H1" is defined as the distance between the upper 15 surface 31c" of the working electrode 31" and the upper surface 32c" of the counter electrode 32".

The present invention is not limited to an analysis tool in which the height of the reaction space is defined by a spacer, and can also be applied to an 20 analysis tool in which the cover is joined to a substrate in which is formed a recess that will serve as the reaction space.

#### Working Examples

25 It will now be proven through Working Examples 1 to 4 that the glucose sensor according to the present invention is capable of measuring glucose concentration

precisely and in a short time, with little effect from blood temperature or hemocytes in the blood in the measurement of response current value.

[Production of Glucose Sensor]

5 In Working Examples 1 to 4, evaluations were conducted using glucose sensors constituted as shown in FIGS. 1 to 3. The glucose sensor used in each working example had a length L (see FIG. 2) of the reaction space 6 of 3.4 mm, a width W (see FIG. 1) of 1.5 mm, 10 and a thickness D (see FIG. 2) of the working electrode 31 and counter electrode 32 of 10  $\mu\text{m}$ . The facing distance H1 in the glucose sensor, the distance H2 between the substrate 3 and the cover 5 (see FIG. 2), and the configuration of the reagent portion 33 were as 15 shown in Table 1 below.

Table 1

	Configuration of glucose sensor		
	Facing distance H1	Height H2 of reaction space	Configuration of reagent portion
Glucose sensor 1 of present invention	23 $\mu\text{m}$	33 $\mu\text{m}$	enzyme-containing layer/electron transport layer
Glucose sensor 2 of present invention	35 $\mu\text{m}$	45 $\mu\text{m}$	enzyme-containing layer/electron transport layer
Glucose sensor 3 of present invention	23 $\mu\text{m}$	33 $\mu\text{m}$	liquid phase electron transport layer alone
Glucose sensor 4 of present invention	44 $\mu\text{m}$	54 $\mu\text{m}$	liquid phase electron transport layer alone

Comparative glucose sensor 1	47 µm	57 µm	enzyme-containing layer/electron transport layer
Comparative glucose sensor 2	47 µm	57 µm	liquid phase electron transport layer alone

With glucose sensors 1 and 2 of the present invention and comparative glucose sensor 1, the reagent portion 33 had a two-layer structure comprising an electron transport layer and an enzyme-containing layer.

5 The electron transport layer was formed by coating the substrate 3 with 0.4 µL of a first material liquid containing an electron mediator, and then drying the coating with forced air (30°C, 10% RH). The enzyme-containing layer was formed by coating the electron 10 transport layer with 0.3 µL of a second material liquid containing a redox enzyme, and then drying the coating with forced air (30°C, 10% RH).

The first material liquid was prepared by mixing the materials numbered (1) to (4) in Table 2 below in 15 that numerical order, allowing this liquid mixture to stand for one to three days, and this added an electron mediator to the mixture. The electron mediator used here was  $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_3$  (LM722 from Dojindo Laboratories).

Table 2

(1) SWN solution		(2) CHAPS solution		(3) Distilled water	(4) ACES solution	
Conc.	Vol.	Conc.	Vol.		Conc.	Vol.
1.2%	250 µL	10%	25 µL	225 µL	200 mM	500 µL

In Table 2 and elsewhere, SWN stands for Lucentite SWN, CHAPS stands for 3-[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid, and ACES stands for N-(2-acetamido)-2-aminoethanesulfonic acid. The SWN used here was "3150" made by Co-Op Chemical, the CHAPS was "KC062" made by Dojindo Laboratories, and the ACES was "ED067" made by Dojindo Laboratories. The ACES solution was adjusted to a pH of 7.5.

10 Meanwhile, the second material liquid was prepared by dissolving a redox enzyme in 0.1% CHAPS. CyGDH (with a glucose dehydrogenation activity of 800 U/mg) was used as the redox enzyme. CyGDH has already been discussed above.

15 In contrast, with the glucose sensors 3 and 4 of the present invention and comparative glucose sensor 2, potassium ferricyanide and potassium ferrocyanide were both present in the reagent portion 33. The purpose of this was to determine more purely the effect that the 20 height of the facing distance H1 has on reproducibility, by excluding the effect of the catalytic function of the redox enzyme and other such factors. More specifically, the reagent portion 33 was formed as a liquid phase by holding a liquid material on the 25 substrate 3. The liquid material used here was prepared so as to contain 20 mM potassium ferricyanide, 24 mM potassium ferrocyanide, and 1.5 M potassium

chloride.

Working Example 1 (Investigation of effect of hematocrit value)

5 In this working example, the effect that the hematocrit (Hct) value has on the response current value was evaluated using the glucose sensors 1 and 2 of the present invention and comparative glucose sensor 1.

10 The blood used in this evaluation had a glucose concentration of 412 mg/dL and a Hct value of either 19%, 42%, or 69%.

15 The application of voltage between the working electrode 31 and the counter electrode 32 was commenced simultaneously with the supply of blood, with the applied voltage set at 200 mV. The response current value was measured 5, 7, and 10 seconds after the start of the voltage application. The response current value was measured five times for each blood Hct value.

20 The results of measuring the response current value are shown in FIG. 7 for glucose sensor 1 of the present invention, FIG. 8 for glucose sensor 2 of the present invention, and FIG. 9 for comparative glucose sensor 1. In FIGS. 7 to 9, the horizontal axis is time 25 (seconds) and the vertical axis is the bias (%). The bias (%) indicates the amount of deviation from a reference value, when the response current value at a

Hct value of 42% was used as the reference value. In each graph, the bias (%) indicates the average of five measurements.

As can be seen from a comparison of FIGS. 7 to 9, 5 regardless of the voltage application time, the bias tends to decrease in proportion to the facing distance H1. Therefore, the smaller is the facing distance H1, the less effect the Hct value of the blood tends to have.

10

#### Working Example 2 (Effect of temperature)

In this working example, the effect that the blood temperature has on the response current value was evaluated using the glucose sensors 1 and 2 of the 15 present invention and comparative glucose sensor 1.

The blood used in this evaluation had a Hct value of 42% and a glucose concentration of either 100.0 mg/dL, 422.0 mg/dL, or 636.0 mg/dL, and its temperature was either 5°C, 25°C, or 45°C.

20 The application of voltage between the working electrode 31 and the counter electrode 32 was commenced simultaneously with the supply of blood, with the applied voltage set at 200 mV. The response current value was measured 5 seconds after the start of the 25 voltage application. The response current value was measured five times for each blood glucose concentration.

The results of measuring the response current value are shown in FIG. 10 for glucose sensor 1 of the present invention, FIG. 11 for glucose sensor 2 of the present invention, and FIG. 12 for comparative glucose 5 sensor 1. In FIGS. 10 to 12, the horizontal axis is temperature (°C) and the vertical axis is the bias (%), shown individually for each glucose concentration. The bias (%) here indicates the amount of deviation from a reference value, when the response current value at a 10 temperature of 25°C was used as the reference value. In each graph, the bias (%) indicates the average of five measurements.

As can be seen from a comparison of FIGS. 10 to 12, regardless of the glucose concentration and voltage 15 application time, the bias tends to decrease in proportion to the facing distance H1. Therefore, the smaller is the facing distance H1, the less effect the blood temperature tends to have.

20 Working Example 3 (Evaluation of measurement range)

In this working example, the measurement range was evaluated using glucose sensor 1 of the present invention. The measurement range was evaluated from the relationship (linearity) between glucose 25 concentration and response current value.

The blood used in this evaluation had a Hct value of 42% and a glucose concentration of either 0 mg/dL,

100 mg/dL, 200 mg/dL, 400 mg/dL, 610 mg/dL, 805 mg/dL, or 980 mg/dL. The application of voltage between the working electrode 31 and the counter electrode 32 was commenced simultaneously with the supply of blood, with 5 the applied voltage set at 200 mV. The response current value was measured 3 seconds after the start of the voltage application. The response current value was measured ten times for each blood glucose concentration.

10 The results of measuring the response current value are shown in FIG. 13. In FIG. 13, the response current value ( $\mu$ A) indicates the average of ten measurements.

15 As can be seen from FIG. 13, glucose sensor 1 of the present invention exhibits high linearity within a glucose concentration range of 0 to 1000 mg/dL, so we can conclude that glucose concentration can be properly measured even when the glucose concentration is relatively high (600 mg/dL or higher). Therefore, if a 20 ruthenium complex is used as the mediator and CyGDH is used as the redox enzyme, as with glucose sensor 1 of the present invention, we can conclude that the concentration of glucose can be properly measured over a range of 0 to 1000 mg/dL in a short measurement time 25 of about 3 seconds.

Working Example 4 (Evaluation of reproducibility)

In this working example, the reproducibility of the response current value was evaluated on the basis of the time course of the relative standard deviation 5 C.V. (%) and the time course of the measurement of the response current value a number of times using the glucose sensors 3 and 4 of the present invention and comparative glucose sensor 2.

The blood used in this evaluation had a Hct value 10 of 42% and a glucose concentration of 412 mg/dL. The application of voltage between the working electrode 31 and the counter electrode 32 was commenced simultaneously with the supply of blood, with the applied voltage set at 200 mV. The response current 15 value was measured 5 seconds after the start of the voltage application. The response current value was measured every 50 msec after the start of voltage application.

The time course measurement results are shown in 20 FIGS. 14 to 16. In these graphs, the time courses of the response current value in five measurements are shown at the same time, with FIG. 14 showing the results when glucose sensor 3 of the present invention was used, FIG. 15 when glucose sensor 4 of the present 25 invention was used, and FIG. 16 when comparative glucose sensor 2 was used. FIG. 17 shows the time course of the C.V. (%). This time course was produced

on the basis of measuring the response current value five times for obtaining a response current value time course.

As can be seen from FIGS. 14 to 16, with glucose sensors 3 and 4 of the present invention, almost no variance is seen in the time courses of the response current value, just as with comparative glucose sensor 2, and good reproducibility was obtained over a number of measurements. On the other hand, as can be seen from FIG. 17, with glucose sensor 3 of the present invention, soon after the start of voltage application, that is, within a time range of 0.5 to 3.0 seconds from the start of voltage application, the C.V. was smaller than with glucose sensor 4 of the present invention or with comparative glucose sensor 2, and the C.V. value was roughly 2.5% or less. With glucose sensor 4 of the present invention, within a time range of 3.0 to 7.0 seconds from the start of voltage application, the C.V. was smaller than with comparative glucose sensor 2, and the C.V. value was roughly 2.5% or less. It can be seen from these results that if the facing distance H1 is set small, reproducibility will be good over a short time range from the start of voltage application. Therefore, from the standpoint of reproducibility, a glucose sensor whose facing distance H1 is set small can be considered suited to a shorter measurement time.